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**14. ABSTRACT**
The aggressive behavior of malignant breast cancer is determined by a complex array of signaling pathways that regulate cell growth, survival and migration. The PI 3-K-Akt pathway has been linked to all of these responses. The current paradigm states that deregulated PI 3-K-Akt signaling promotes cancer progression. Many of the enzymes that regulate PI 3-K signaling are frequently mutated in human breast cancer, thereby up-regulating Akt activity and increasing tumor cell growth and survival. This is best illustrated by the finding that the catalytic subunit of PI 3-K, PIK3CA, is the most frequently mutated oncogene in breast cancer. However, recent studies have demonstrated that distinct Akt isoforms can either inhibit or enhance breast cancer cell invasive migration and metastasis in vitro and in vivo, probably by phosphorylating a different set of substrates in an isoform specific manner.
Afadin is a Ras target gene that regulates cell-cell adhesion downstream of Ras. Afadin protein loss of expression is associated with poor outcome in breast cancer patients. The most significant finding of this research thus far is that Afadin –isoform 3, which is the longer, ubiquitously expressed form of the protein, is a substrate of Akt, down stream of the PI-3K pathway.

**15. SUBJECT TERMS**
Phosphorylation, proteomic screen, SILAC

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**Table of Contents:**

**Introduction:**

Increased Akt activity is detected in aggressive human breast cancers [1, 2] and is associated with poor prognosis and higher probability of relapse accompanied by distant metastases in patients [3]. Experiments in transgenic mice have revealed that Akt promotes mammary tumor progression by increasing cell survival [4]. Moreover, many of the proteins in the Akt pathway are either oncogenes (e.g. Akt itself, PI 3-K, MDM2) or tumor suppressors (e.g. the PIP3 phosphatase PTEN and Akt phosphatase PH domain Leucine-rich repeat Protein Phosphatase (PHLPP)), and indeed these proteins are frequently deregulated in breast cancer. The significance of the Akt pathway in cancer has made it an attractive target for the development of small molecule inhibitors [5]. There are three mammalian Akt isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). The three Akt isoforms are encoded by distinct genes, but share a high degree of amino acid similarity and are activated by similar mechanisms and recognize and phosphorylate the same consensus motif [6]. The objectives of this research are to discover novel substrates of the PI-3K-Akt pathway that promote malignant phenotype in breast cancer, with a special scope on Akt-isoform specific substrates and Afadin, as a putative novel substrate of PI-3K pathway.

**Body:**
Description of the research accomplishment associated with the tasks outlined in the approved statement of work:

**Aim1:Afadin Regulates Breast Cancer Cell Migration Downstream of Akt and Serum glucocorticoid induces Kinase (SGK)**.
Aim1 task1: Validate the phosphorylation of Afadin-i3 as a substrate of the Phospho -Inositide 3 Kinase (PI 3-K) pathway in breast cancer cells. (time frame months 1-12) This task is complete, Afadin-i3 was found to be a substrate downstream of the PI-3K signaling pathway.
Subtask 1a: Determine if AF6i3 S1718 is phosphorylated by a kinase downstream of the PI 3-K pathway using chemical inhibitors that inhibit different components of the PI 3-K pathway. (time frame months 1-4). This subtask is complete.
I used different pharmacological inhibitors to find out whether Afadin-i3 is a substrate downstream of PI-3K pathway. Different breast cancer cell lines including MCF10A and MDA-MB-231 were grown to high confluency in full serum and than serum starved for 18 hours. The cells were treated with the inhibitors for 20 minutes. The inhibitors and concentration that were used in this experiment:
Wortmannin (100nM) - PI 3-K inhibitor
LY294002 (100uM) – PI 3-K inhibitor
SN30798 (5uM) – Akt inhibitor
BEZ235 (5uM) - PI 3-K and mTOR inhibitor
Rapamycin - Torc1 inhibitor
GSK650394 (10uM) – SGK inhibitor
PP242 (2.5uM) – mTor inhibitor
PF4708671 (10uM) – p70 ribosomal S6 Kinasw (S6K) inhibitor
After treatment with the inhibitors cells were stimulated with IGF1 (100ng/ml, 20 minutes) in order to stimulate the PI-3K pathway. Cells were harvested using RIPA buffer and subjected to Western Blotting (Figure1).

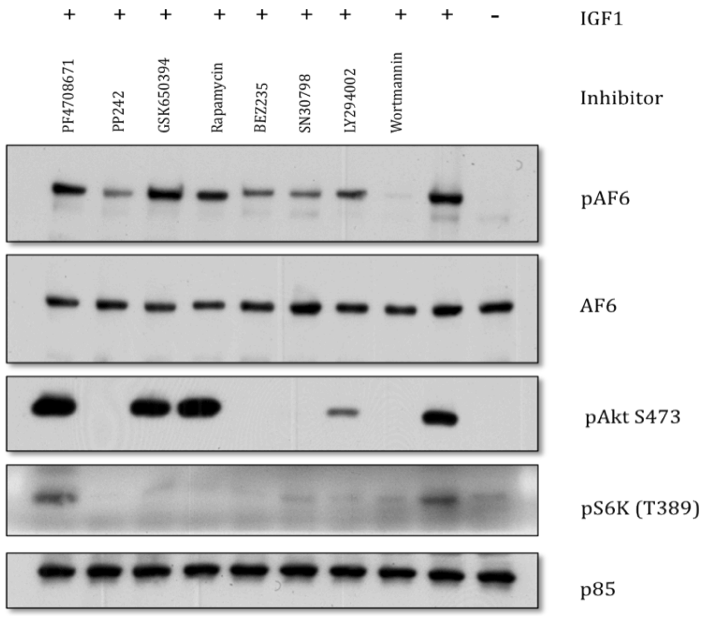


**Figure 1:** MCF10A cells treated with the indicated inhibitors and stimulated with IGF1.

In order to evaluate the phosphorylation of AF6 I used a specific phospho S1718 AF6 antibody (pAF6). In order to assess the pathway stimulation status after treatment with inhibitors or IGF1 alone I used phospho S473 Akt antibody (S473) as well as phospho T389 S6K antibody (as a readout of PF4708671 activity). As described in figure 1, AF6i3 was found to be a substrate of Akt and not SGK or S6K, downstream of PI-3K pathway. P85 was used as a loading control.
Subtask 1b: Determine what kinase phosphorylates AF6i3 S1718 using isoform specific shRNAs. (time frame months 5-8). This subtask is complete.
I used specific shRNA sequences targeting the different Akt isoforms. These sequences were cloned into the pLKO vector. 293T cells were transfected with these shRNAs and packaging vectors in order to generate lentivirus to infect breast cancer cells. Different breast cancer cell lines were infected with the lentivirus and selected with puromycin. In this process, specific Akt isoforms were silenced. Cells were grown to high confluency in full serum and than serum starved for 18 hours. The cells were either stimulated with IGF1 (100ng/ml) for 20 min or not treated with IGF1. Cells were harvested and subjected to Western blotting.
The antibodies that were used in this experiment were phospho S1718 Afadin (pAfadin), total Afadin and specific antibodies against Akt1 and Akt2. Due to the lack of a good Akt3 antibody, the blot is not presented here. P85 was used as a loading control (Figure2).

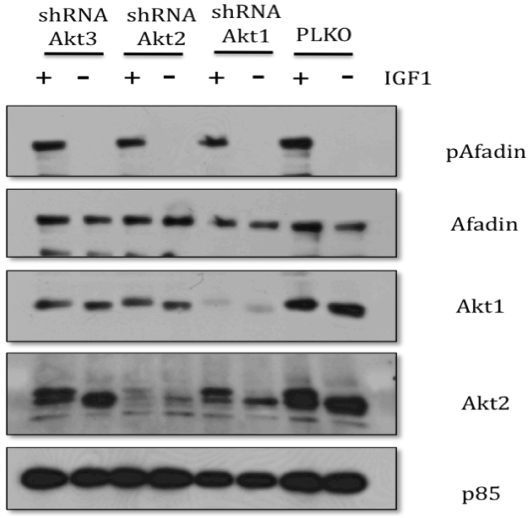


**Figure 2:** MCF10A cells were silenced with specific shRNA against Akt isoforms.

Subtask 1c: Determine what kinase phosphorylates AF6i3 S1718 using wild type and active alleles of Akt1/2/3 SGK1/2/3. (time frame months 5-8). This subtask is complete.
Different breast cancer cell lines were transfected with the constitutively activated alleles of the different Akt isoforms. 24 hours after transfection cells were serum starved for 18 hours and harvested. In order to evaluate the role of the activated alleles, cells were just serum starved and not stimulated with any growth factor, that way, the phosphorylation that is seen under these conditions would be attributed only to the activated alleles. The activated Akt alleles are activated by myristoylation. As a negative control I transfected the cells with pcDNA3 vector. AF6i3 was found to be phosphorylated by all three Akt isoforms, and not by SGK isoforms as can be seen by using the inhibitors in figure 1 and by using constitutively active alleles in figure 3.

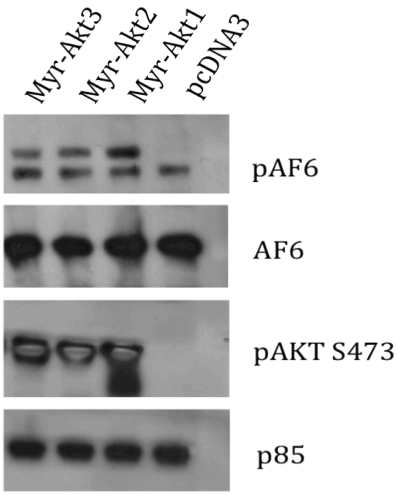


**Figure 3:** MCF10A transfected with activated Akt alleles.

Subtask 1d: In vitro kinase assays. (time frame months 9-12). This subtask is complete.
Cells were transfeted with wild type AF6i3 or with the S1718A mutant of AF6i3 (a vector which contains a mutant phosphorylation site, that cannot be phosphorylated). The AF6i3 plasmid also contains a myc tag, that was used for immunoprecipitation (IP). The protein that were IP'ed from the cells (either with wild type or mutant phosphosite) were incubated with recombinant Akt1 or Akt2 kinases in 30$^{o}$C for 60 minutes. I also transfected the cells with a well-known substrate of Akt, Glycogen Synthase Kinase 3-beta (GSK-3beta). GSK3beta protein was IP'ed using the HA tag in the plasmid. Just like AF6i3, the protein was incubated with recombinant Akt1 and Akt2. Samples were subjected to Western Blotting (Figure 4). By In vitro Kinase assay, I was able to show that Akt specifically phosphorylates the Serine in 1718 site of AF6i3. Cells that were transfected with GSK-3-beta were used as control for the ability of the recombinant Akt1 and Akt2 to serve as kinases in vitro.

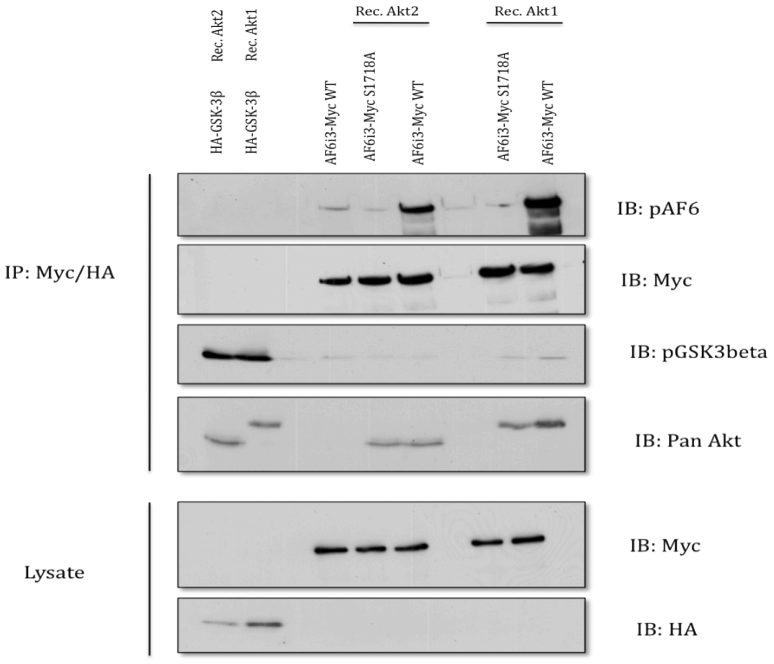


**Figure 4:** In vitro kinase assay.

In order to evaluate the phosphorylation status of AF6i3 alleles, I used pAF6 antibody. The results show that AF6 is phosphorylated in S1718 by Akt. pGSK3beta antibody was used to check the activity of Akt kinases. In order to assess the amount of recombinant Akt in these samples I used pan Akt antibody, and the amount of AF6 (different mutants) or GSK3beta were evaluated using the Myc and Akt tags.

**Aim 2: Phospho-proteomic analysis of Akt1/2/3 and SGK1/2/3 substrates in breast cancer**
Aim 2 task 1: Cloning and validating Tet-On shRNA vectors. (time frame months 1-8). This task is complete.
Subtask 1a: Cloning of pLKO Tet-On shRNA vectors. (time frame months 1-4). This subtask is complete. I was able to clone the shRNAs into the pLKO Tet-On vectors. In order to do so the pLKO-Tet On vector was digested using restriction enzymes, the two shRNA sequences (sense and antisense) were annealed and than phosphorylated. The digested vector was ligated to the annealed phosphorylated oligos over night. The ligate DNA was transformed into bacteria, the next day I chose a few colonies, prepared DNA from them, screened them for the insertion of shRNA sequences in PCR using specific primers, and in order to make sure the chosen colonies had the right sequences, these DNAs were sequenced.
Subtask 1b: Validating if pLKO Tet-On shRNA vectors work and silence target mRNAs using Doxycycline. (time frame months 5-8). 293T cells were transfected with the PLKO-Tet-On shRNAs and packaging vectors in order to generate lentivirus to infect breast cancer cells. Different breast cancer cell lines were infected with the lentivirus and selected with puromycin. In this process, specific Akt isoforms were silenced. Cells were grown to high confluency in full serum and than serum starved for 18 hours. The cells were either treated or not with Doxycycline (Dox) (100ng/ml) for 48 hours in order to stimulate shRNA production in the cells treated with Dox. Cells were harvested and subjected to Western blotting. The validation was done on all cell lines; a representative sample of MDA-MB-231 cells is shown in Figure 5.

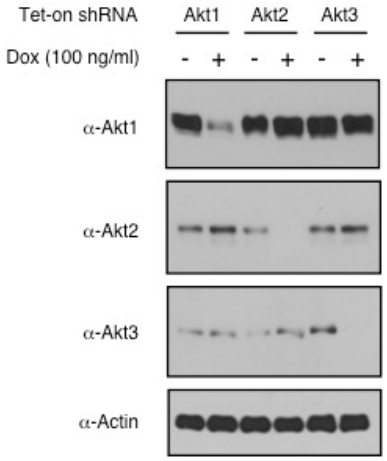


**Figure 5:** silencing of Akt isoforms in MDA-MB-231

In order to assess silencing of specific Akt isoforms I used antibodies targeting the different Akt isoforms (Akt1, Akt2 and Akt3) Actin served as a loading control. Silencing of Akt isoforms is specific following Dox treatment.

Subtask 1c: Culturing of the breast cancer cell lines used for this phospho-proteomic analysis and determining Akt1/2/3 and SGK1/2/3 basal expression level. (time frame months 5-8). The results for Akt and SGK3 expression levels in the cell lines are presented in Figure 6. SGK2 was not expressed in any of the cells lines that are tested in this screen. As for SGK1 expression, up until now I wasn't able to find any good antibody that will enable me to determine the expression levels of SGK1 in these cell lines. I was able to overcome this problem by doing an RT-PCR, but since the first part of this screen is to determine SGK1 protein levels, the unavailability of a good SGK1 antibody has led me to focus my effort in this screen in Akt isoforms as well as SGK3. Moreover, a paper that was recently published suggests that many PIK3CA mutant cancer cell lines and tumors exhibit only minimal Akt activation and a diminished reliance on Akt for anchorage-independent growth. Instead, in these cells and tumors SGK3 undergoes PI3K- and PDK1-dependent activation [7]. Figure 6 demonstrates the expression levels of Akt isoforms and SGK3 in 2 breast cancer cell lines: MDA-MB-468 and BT-549. I chose to begin working on these cell lines because they have pathway activation mutation in the PI-3K pathway, in both cell lines PTEN is mutated, which leads to hyper activation of the pathway.

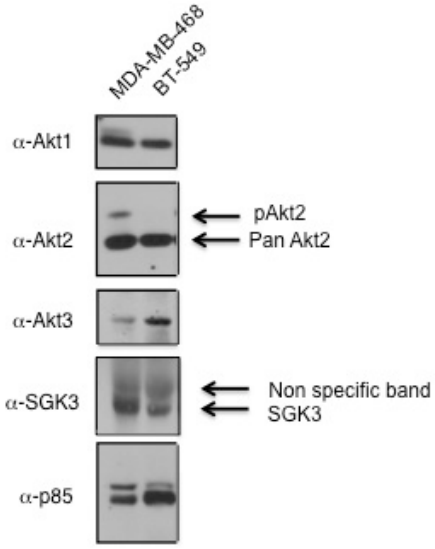


**Figure 6:** expression levels of Akt isoforms and SGK3 in MDA-MB-468 and BT-549

Aim 2 task 2: Silencing of Akt1/2/3 and SGK1/2/3. (time frame months 9-15).
Subtask 2a: Infection of breast cancer cell lines. (time frame months 9-12). This subtask is complete.
Subtask 2b: Generating stable clones from each shRNA vector in each cell line. (timeframe months 9-15). Stable clones of the pLKO Tet-On expressing cells were generated. 293T cells were transfected with the shRNAs and packaging vectors in order to generate lentivirus to infect breast cancer cells. Different breast cancer cell lines were infected with the lentivirus and selected with puromycin. Depending on the cell lines and their ability to proliferate from a single cell population, cells were either cloned or kept as a heterogeneous pool of infected selected cells. Cells were grown in full serum (the serum did not contain traces of tetracycline). The cells were treated with Dox for 48 and 72 hours and serum starved for 18 hours. Cells were harvested and subjected to Western blotting in order to check the efficiency of silencing.
An example of stable clones expressing the pLKO Tet On shRNA for Akt1, Akt2 and Akt3 in MDA-MB-468 is presented on Figure 7. Due to a poor Akt3 antibody at the time of the

experiment, the efficiency of Akt3 silencing was check with RT-PCR. I realize that this is not the most accurate way of measuring protein levels, but at the time of the actual experiment this was the best tool available. In general, cells expressing the pLKO-Tet On system and treated with Doxycycline, do not inhibit protein expression as good as they would if infected with pLKO system (non-inducible). This phenomenon is seen with almost every cell line tested. In the next year we will have to find a way to overcome this difficulty and/or to modify our experiment.

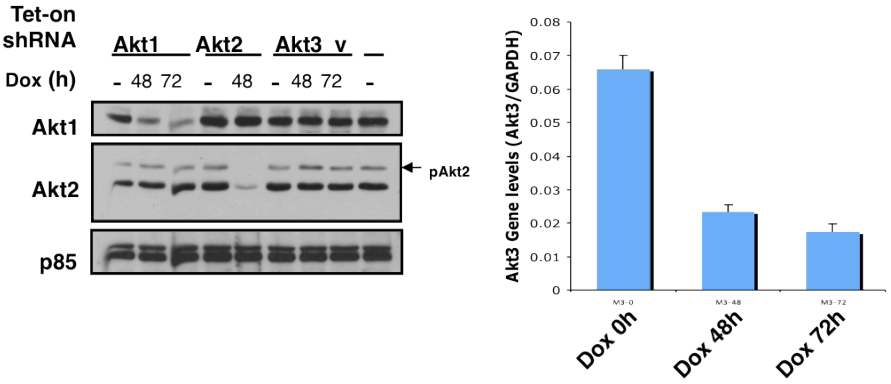


**Figure 7:** Akt isoforms were silenced using pLKO Tet-On system in MDA-MB-468 cells

Subtask 2c: Perform Stable Isotope Labeling by Amino acids in Cell culture (SILAC) experiment. (timeframe months 12-15). This subtask was performed on some of the cell lines, to check if this method is applicable. The cells we chose to start with were MDA-MB-468. SILAC experiment was performed on this cell line and they were sent to Cell Signaling Technology for Mass Spec analysis.

**Key Research Accomplishments:**

- Afadin isoform 3 was found to be a novel substrate of Akt in the PI-3K signaling pathway.
- The phosphorylation site is S1718 in Afadin isoform 3.
- Afadin isoform 3 was found to be a substrate of all three Akt isoforms.

**Reportable Outcomes:**
During the reported period I presented posters in two meetings:

1. Keystone Symposia on Molecular and Cellular Biology – PI-3K Signaling Pathways – the meeting took place in Keystone, CL in February 13-18, 2011 (Appendix1).
2. Era of Hope Meeting of Breast Cancer Research Program (BCRP), Congressionally Directed Medical Research Program (CDMRP), Department of Defense (DOD), this meeting took lace in Orlando, FL in August 2-5, 2011 (Appendix 2).

My training program in the last year included weekly meetings with Dr. Toker, my postdoctoral mentor, where we analyzed results and plan experiments. In addition, our laboratory meets once a week for a lab meeting. In these meetings we receive feedback on our work, as well as advice and input. In the last year I presented my project 6 times in lab meetings. Moreover, the Toker laboratory has a bi-weekly journal club meeting where discussions focus on recently published literature in the breast cancer field. I presented twice in the journal club and participated in all of the meetings we had. Finally, as part of the various intramural training activities within the laboratory, our lab attends a weekly cancer signaling meeting at Harvard Medical School. These meetings are designated to refine students' and postdocs' presentation skills. These meetings allow the participants and speakers to receive feedback on work from leaders in their respective fields, including Lewis Cantley, Joan Brugge and John Blenis. In the reported year I presented once in this forum.

**Conclusions:**

In the reported year I was trying to find out whether Afadin isoform 3 is a novel substrate of the PI-3K pathway. I found that it is a substrate of Akt. The phosphorylation site is S1718. I was trying to find out whether it's an Akt isoform specific substrate, in this part I found that it's a general substrate that can be phophorylayted by all three Akt isoforms but not by other AGC kinases. In the last few months I'm trying to establish the biological significance of this phosphorylation, and to reveal the connection between PI-3K and Akt to this important adhesion protein.

With the proteomic screen, I have been trying to silence the expression of a specific kinase by the pLKO-Tet-On system. Lately the first round of cells were sent to Cell Signaling Technology to complete the mass spec experiment, and we hope to receive some initial results soon. If necessary, I might have to modify the protocol and try to get better, more significant results. The main goal of this part of the research is to find novel isoform specific substrates of Akt that would help explain the importance of this family of kinases in many processes in breast cancer.

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## Appendices:

1.

**Identification of Novel Isoform-Specific Substrate in the PI 3-K and Akt Signaling Pathway**

**Sivan Elloul and Alex Toker, Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA.**

The aggressive behavior of malignant breast cancer is determined by a complex array of signaling pathways that regulate cell growth, survival and migration. The PI 3-K-Akt pathway has been linked to all of these responses. The current paradigm states that deregulated PI 3-K-Akt signaling promotes cancer progression. Many of the enzymes that regulate PI 3-K signaling are frequently mutated in human breast cancer, thereby up-regulating Akt activity and increasing tumor cell growth and survival. This is best illustrated by the finding that the catalytic subunit of PI 3-K, *PIK3CA*, is the most frequently mutated oncogene in breast cancer. The essential role of Akt in cancer progression has made it a very attractive target of inhibition in cancer therapy. However, recent studies have demonstrated that distinct Akt isoforms can either inhibit or enhance breast cancer cell invasive migration and metastasis *in vitro* and *in vivo*. While Akt1 blocks epithelial migration in breast cancer cells, Akt2 does not block or even enhances epithelial migration, which can lead to metastatic dissemination. More then 150 substrates of Akt have been characterized to date, yet only a handful have been shown to be isoform-specific and only one of these, palladin, an Akt1 specific substrate, has been shown to transduce the Akt signal to epithelial invasive migration in breast cancer. We are using a phospho-proteomic screening approach to identify novel Akt substrates that modulate invasive migration and metastasis in breast cancer. Using this approach we have identified Afadin/AF6 as a novel Akt substrate. Afadin/AF6 is a tumor suppressor-like protein that connects nectin to the actin cytoskeleton and helps to maintain the architecture of adherens junction in epithelial cells. Loss of Afadin protein expression is associated with poor prognosis in breast cancer patients. We find that Afadin is phosphorylated at an optimal Akt consensus motif. Afadin phosphorylation by Akt affects protein stability and lead to degradation. Using gain of function and loss of function approaches in combination with activated and silencing of Akt isoforms as well as specific chemical inhibitors we are investigating the function of Afadin and Akt phosphorylation in breast cancer invasive migration.

**2.**

**Identification of a Novel Substrate in the PI 3-K and Akt Signaling Pathway**

**Sivan Elloul and Alex Toker, Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA.**

The aggressive behavior of malignant breast cancer is determined by a complex array of signaling pathways that regulate cell growth, survival and migration. The phosphoinositide-3-kinase (PI 3-K)-Akt pathway has been linked to all of these responses. The current paradigm states that deregulated PI 3-K-Akt signaling promotes cancer progression. Many of the enzymes that regulate PI 3-K signaling are frequently mutated in human breast cancer, thereby up-regulating Akt activity and increasing tumor cell growth and survival. This is best illustrated by the finding that the catalytic subunit of PI 3-K, *PIK3CA*, is the most frequently mutated oncogene in breast cancer. The essential role of Akt in cancer progression has made it a very attractive target of inhibition in cancer therapy. However, recent studies have demonstrated that distinct Akt isoforms can either inhibit or enhance breast cancer cell invasive migration and metastasis *in vitro* and *in vivo*. While Akt1 blocks epithelial migration in breast cancer cells, Akt2 does not block or even enhances epithelial migration, which can lead to metastatic dissemination. More then 150 substrates of Akt have been characterized to date, yet only a handful have been shown to be isoform-specific and only one of these, palladin, an Akt1 specific substrate, has been shown to transduce the Akt signal to epithelial invasive migration in breast cancer. We are using a phospho-proteomic screening approach to identify novel Akt substrates that modulate invasive migration and metastasis in breast cancer. Using this approach we have

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